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RESEARCH ARTICLE

A preliminary screening of Silk protein (sericin) for the anti-Leishmanial properties and effect on immune reponse in visceral leishmaniasis

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Abstract

Sericin, a silk protein (SP), is one of the main constituents of silk cocoons, comprising 20-30% of total cocoon weight.. In this study, we investigated the anti-leishmanial and immunogenic properties of Sericin from the VL endemic area of Bihar, India and its role on Leishmania donovani (Ld) growth in vitro and MIF release by T-cells of patients was scrutinised . The study identified an important role of serecin as at 10µg/ml it demonstrated significant killing of *L.donovani* promastigote in culture. At this concentration, it was was observed to be less toxic on human peripheral blood mono nuclear cells in comparison to other groups and other concentrations. In further studies it was shown that serecin at threshold concentration of 10 ug can trigger the T cell response for an effective immune response against *L.donovani.*

The present study provides scientific data that support the protective effect of silk protein (sericin) against visceral leishmaniasis (VL) control.

INTRODUCTION

Leishmaniasis is a diverse group of clinical symptoms caused by Leishmania, a prozoan parasite. One of the form of disease caused by Leishmania is Kala-azar, where the primary target of infection is the Bone marrow, Spleen and Liver. It is typically caused by *L.Donovani* complex, which includes three species *L. donovani* (Indian sub constituent and East Africa), *L.infantum* (Mediterranean basin) and *L.chagassi*(Latin America). The disease is transmitted by Sand fly, which inoculates the flagellated promastigotes into the skin of the host. In humans, these promastigote are taken up by macrophages and transform into non-flagellar amastigotes. More than 20,000 cases of VL are reported annually in Indian alone (1-3). The disease is fatal if left untreated. The therapeutic options for controlling Leishmaniasis are limited to a few drugs with inconsistent efficacy and side effects. Thus, there is an urgent need to develop, alternative ways of disease control. There are clear indications that 'Th^{1}' response of CD4^{+} T cells is protective and easy to be induced in these patients. The identification and evaluation of several components which preferentially stimulate protective immune response against Leishmania, may be vital to develop a control strategy against this disease.

Sericin is a silk protein woven from silkworm cocoons (Antheraea mylitta D). The present study investigated the effects of Sericin on human peripheral blood mononuclear cells (PBMCs) compared to normal healthy control. Sericin is a family of adhesive silk protein synthesized in middle silk glands of silkworms [4] that envelops fibroin fibers in cocoon [5]. Usually it constitutes 20–30% of silk protein in cocoon [6-7] and consists of amino acids most of which have strong polar side groups such as hydroxyl, carboxyl, and amino groups with high

serine content contributing to its high hydrophilicity [8]. It is non toxic, antioxidant agent, anti-aging properties like vitamin C [9-11], with antibacterial, UV resistant and anti apoptotic properties [6]. Other biological activities it comprises are anti tyrosinase, anticoagulation and anti-cancerous activities [6-12] such as colon tumorigenesis [1, 13] and supports cell growth and differentiation and has been considered to act as cell culture supplement in serum free media [14]. Sericin also helps in reduction of cholesterol [15]. In this study, we assessed the leishmanicidal activities of sericin against *Leishmania donovani*. Studies on Silk Protein (sericin) extracts were extended to establish their minimum effective concentrations (MECs), their leishmanicidal effects on promastigote and their effects on release of Macrophage migration inhibition facor (MIF) from human peripheral blood mononuclear cells (PBMCs) of active VL patients

2. Materials and methods

2.1 Preparation of crude Silk Protein (sericin) Silk Protein (sericin) was extracted with deionized water from raw silk yarns of the silkworm Antheraea mylitta D under high pressure and high temperature. The Silk Protein extract was later dried at 130 ºC, and then ground and sieved through a 0.75 mm screen. The resulting Silk Protein (sericin) powder was sealed in sterile plastic bags and kept at room temperature until used. The Silk Protein (sericin) was used by reconstituting it in phosphate-buffered saline (PBS) and was sterilized by autoclaving at 121°C for 15 minutes [16].

2.2 Promastigote culture of Leishmania donovani In this study WHO reference strain (MHOM/IN/80/DD8) isolates of L. donovani from the various hospitals of Bihar, India were used. The L. donovani promastigote strains were maintained in RPMI-1640 medium (Sigma-Aldrich, USA) containing 20% heat inactivated fetal bovine serum (FBS; Himedia, India), pH 7.2–7.4, at 24±20C. Cultures were maintained through serial sub-culturing for further studies [17].

2.3. Treatment of L. donovani promastigotes with Silk Protein (sericin) To identify Sericin's anti-leishmanial activity, the effects of SilkProtein (sericin) on the growth on the promastigotes was measured. For these tests, a total of 2x106/ml early stationary phase L. donovani promastigotes (24 h, 48, and 72h culture) in RPMI-1640 complete media with 20% FBS were dispensed into 24-well culture plates. The culture was supplemented with sericin at different concentrations of 5µg/ml, 10µg/ml and 20 µg/ml in duplicate series and cultures were incubated at 24 ± 20C and microscopic analysis was performed after 24h, 48h and 72h (Fig 1) using a 0.1-mm Naubauer Chamber [18].

2.4. Evaluation of minimum effective concentrations of Silk Protein (sericin) showing significant leishmanicidal properties We evaluated the minimum effective concentrations (MECs) of Silk Protein (sericin) that showed significant levels of L. donovani killing. The leishmanicidal activity of Silk Protein (sericin) against L. donovani promastigotes (2x106/ml) were evaluated in duplicate series (Fig.2) at different concentrations of 5µg/ml, 10µg/ml and 20µg/ml. The concentration of the SilkProtein (sericin) was measured at 570nm by spectrophotometer.

2.5 Evaluation of cell toxicity of Silk Protein (sericin) at the MEC against human Peripheral Blood Mononuclear Cells. To evaluate the cytotoxicity of SilkProtein (sericin) against human PBMCs [17-18], the mitochondrial dehydrogenase-based 3-(4,5-diamethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used. To perform this assay, MTT-based in vitro toxicology assay kit (Sigma–Aldrich, USA) was used. Briefly, peripheral blood samples taken from 5 healthy human volunteers and PBMCs were isolated by density gradient centrifugation (2000 RPM, 15 min) over Histopaque-1077 (Sigma, USA). After washing with PBS, PBMCs (1x106cells/ml) were cultured in complete RPMI-1640 with 20% FBS media (without phenol red). Further, 100 µl of MTT solution (10% of PBMCs culture) were added to PBMCs and incubated for 2 h in a CO2 incubator at 35 ºC and >95% humidity. After incubation period these cells were supplemented with 1 ml MTT solubilization solution. These cells were then vortexed to dissolve formazan crystals and analyzed with spectrophotometer at a wavelength of 570 nm, using 690 nm as reference. Values obtained using SilkProtein (sericin) treated cultures were analyzed. Mitochondrial dehydrogenases cleave the tetrazolium ring of MTT, in viable cells and yielded insoluble purple formazan crystals. The higher concentration of formazan is indicative of a high level of mitochondrial dehydrogenase activity [17].

2.6 Evaluation of release of Macrophage migration inhibition factor (MIF) from T-cells

MIF was measured in all subjects as an index of cell mediated immunity (Clausen, 1971). In brief, wells were cut in a preparation of agarose in a petri dish (15 · 90 mm) for a reaction of cultured lymphocyte cell suspension in the

presence and absence of mouse antihuman CD2 antibody.After 24h incubation at 5% CO2 in air at 37 _C, further incubation of MIF plates was stopped and theT-cell reactivity was assessed by measuring MIF, a glycoprotein lymphokine in percentage value, after fixation and staining in 0.37% formaldehyde, and gentian violet suspension. The diameter of the migration areas was measured by ocular meter and the percentage of migration inhibition was calculated as follows:

$$\text{Migration inhibition} = 1 - \frac{\text{mean migration diameter of cells in the presence of antigen}}{\text{mean migration diameter of cells in the absence of antigen;}}$$

$$\text{Percentage migration inhibition} = \text{Migration inhibition} \times 100$$

2.6 Statistical Analysis

The results were expressed as the mean ± SD unless otherwise indicated

3. Results and Discussion

The effect of Silk Protein (sericin) on the growth pattern of L.donovani was determined. Out of 5µg/ml, 10µg/ml and 20µg/ml, only 10µg/ml at 48h had shown maximum killing of *L.donovani* promastigote (Fig.1).In subsequent experiments, Cell toxicity of Sericin at the MEC against human Peripheral Blood Mononuclear cells was also evaluated to analyze the safety of the SP (sericin) as having anti-leishmanial properties. It was observed that Silk Protein (sericin) showed the maximum effect against promastigotes in culture at 48h and 10µg/ml. At this concentration, it was accompanied with maximum decline in dehydrogenase activity of mitochondria and was observed to be less toxic in comparison to other groups and other concentrations. We subsequently investigated the T-cell response in response in presence and absence of serecin on VL patients. The initial trend revealed that in general, VL patients in their active stage of disease were accompanied by an insignificant secretion of MIF by T-cells (Table 1). It was apparent from this finding that low number of T cells produced MIF , so they were unable to trigger activation events in T-lymphocyte for active cytokine response to *L. donovani* antigen. Previously, Bimal et al. (2005) reported a reduction in MIF release by sub-population of T-cells in Indian patients. As a result, T-cells were not stimulated and MIF response was very low. After stimulation of PBMCs with serenin, there occurred an increase in MIF secretion by these cells. These findings indicate that serecin at threshold concentration of 10 ug can trigger the T cell response for an effective immune response against *L.donovani*.

Silk protein (Sericin) are of considerable interest of anti-leishmanial properties. Previously Silk protein (sericin) has been reported to suppress colon tumorigenesis in animal models [20-21] and many other important roles like antioxidant agent, non-toxic [9,10], antibacterial [6], reduction in cholesterol [14] and cell culture supplement in serum free media [13]. However, there is still a limited number of studies that explain SP (sericin's) role in treatment of visceral leishamaniasis. SP has anti-leishmanial effect that will help in the effective treatment of visceral leishmaniasis. This present study gives new insight whether SilkProtein (sericin) has any effects on VL in Indian kala-azar patients.

To further determine the role of serecin, we examined the production of MIF by exposing the T-cells of patients in vitro to agnostic anti-CD2 mAb. We preferred MIF on account of its simplicity, ease of use in laboratories with limited facilities and its relative sensitivity when compared to other cytokine based detection assays such as interferon-c (IFN-γ) evaluation, which are generally less affordable in remote rural areas where modern methods cannot be used(Bimal et al,2005). It was observed that patients with active infection potentially generated more MIF in presence of serecin, which was secreted less in absence of serecin exposure. This study identifies a crucial role of serecin against L. donovani in VL.

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Table 1

Effect of Sericin on release of MIF by T cell in VL patients

Figure 1. Treatment of L. donovani promastigotes with S i l k P r o t e i n (sericin).

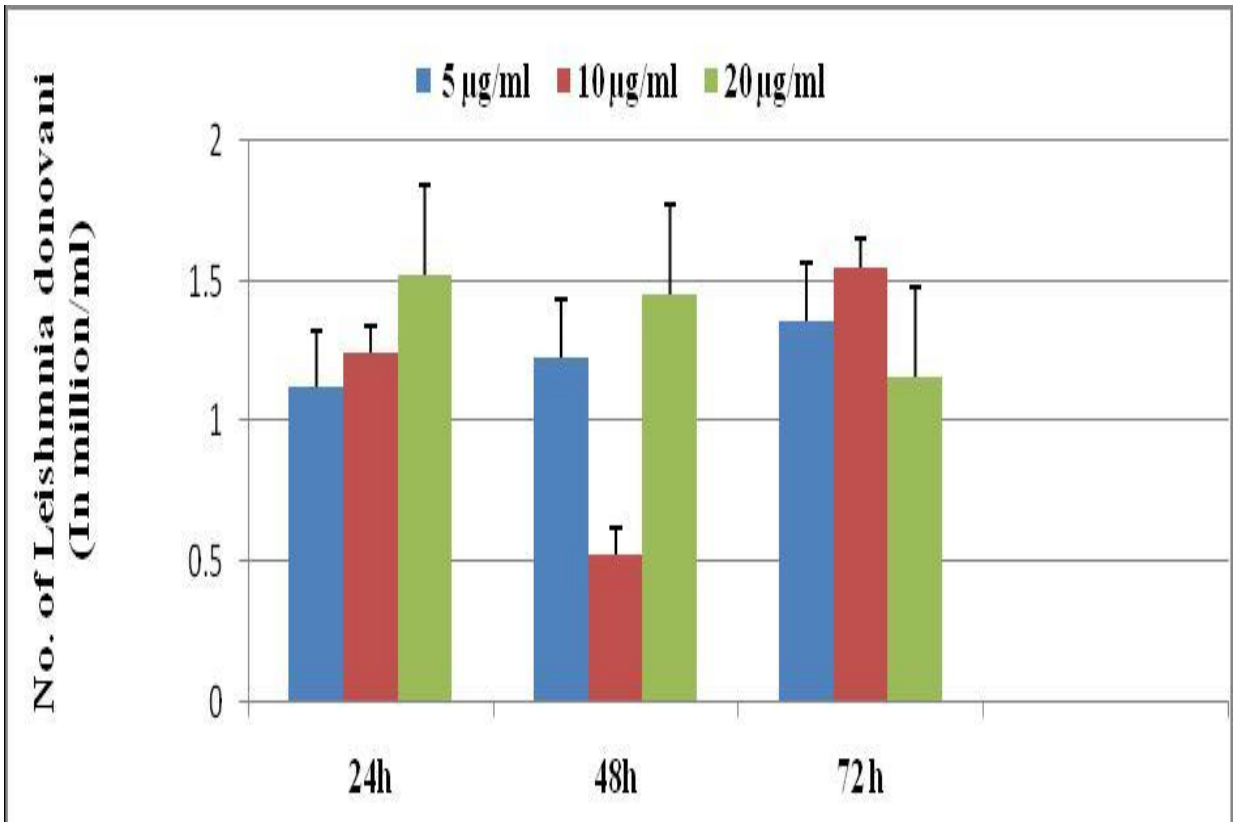

Figure 2 Evaluation of minimum effective concentrations of Silk Protein (sericin) showing significant leishmanicidal properties

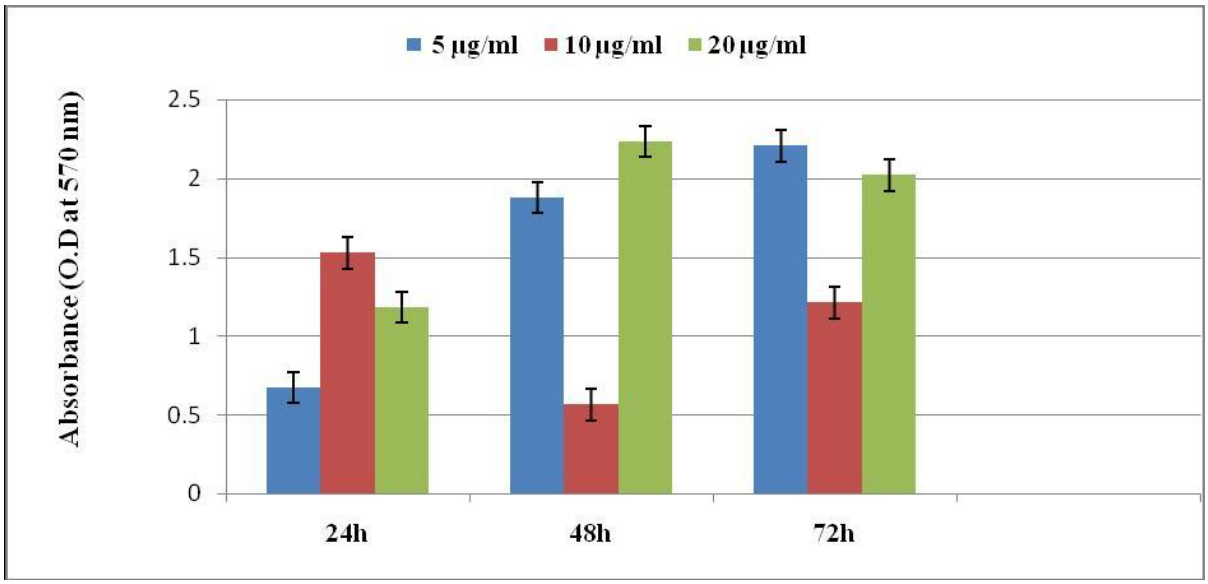

Table 1

Effect of Sericin on release of MIF by T cell in VL patients

Category	MIF (%)	Sericin	PHA
	Unstimulated	Stimulated	Stimulated
Untreated VL (n=10)	16.88±4.56	27.62±3.26	37.31±6.89
Healthy control (n=10)	25.57±3.36	33.36±6.51	35.63±6.80

Table showing effect of Sericin during active VL infection with strong T-helper cell response for MIF release, which increased when MNC were stimulated with 10μg Sericin.

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